



US Environmental Protection Agency Office of Pesticide Programs

**Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD**

**Germicidal and Detergent Sanitizing Action of
Disinfectants Test**

SOP Number: MB-27-04

Date Revised: 12-16-22

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Title	Germicidal and Detergent Sanitizing Action of Disinfectants Test
Revisions Made	<ul style="list-style-type: none"> • Minor editorial changes for clarification purposes. • Added language to section 11. Special Apparatus and Materials as necessary to further clarify purpose of medium or reagent. • Deleted “PBDW-SS” from section 11.4 because it is the same reagent as “PBSS”. • Removed the statement “For use with ≤ 200 ppm quaternary ammonium compounds from section 11.4.c Neutralizer blanks. This statement is not present in the most recent version (2013) of AOAC 960.09. • Added “PBS 10X Solution” to section 11.4. • Added “and associated water bath” to section 11.5.b. Recirculating Chiller Unit to further clarify the requirement. • Removed the statement “Note: A new frozen stock culture may be initiated one time using an existing, unexpired frozen stock culture as the source” from section 12.1 Culture Preparation. For AOAC methods, frozen stock cultures must be initiated every 18 months using a purchased lyophilized culture. • Added “(i.e., chiller unit display)” to section 12.3.e, “Record the temperature of the water bath and recirculating chiller” to clarify parameter to record. • Revised Form 3 to create a field to record chiller/water bath temperature information (i.e., chiller unit display and water bath, before and after testing). • Relocated Neutralization Confirmation Test from section 12.7 to section 12.9, after results and confirmatory steps sections for better flow. • In section 12.8 Confirmatory Steps for Test Microbes, changed incubation time from 18-24 hours to 24 ± 2 hr to bring section in line with other SOPs and Attachment 2 of this SOP. • Combined references in section 15 into one reference for both <i>S. aureus</i> and <i>E. coli</i>.

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Title	Germicidal and Detergent Sanitizing Action of Disinfectants Test
Scope	Describes the methodology to determine the efficacy of food contact sanitizers against <i>Staphylococcus aureus</i> and <i>Escherichia coli</i> . The test is based on AOAC method 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants – revision date 2013.
Application	The methodology described in this SOP is used to evaluate the performance of food contact sanitizers against the prescribed test microbes.

	Approval	Date
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1. Definitions	Abbreviations/definitions are provided in the text.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with test chemicals.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOPs EQ-01 (pH meter), EQ-02 (Thermometer), EQ-03 (Weigh Balance), EQ-05 (Timers), and QC-19 (pipettes).
5. Sample Handling and Storage	Refer to SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	No contamination is acceptable in the treated or numbers control plates.
8. Non-conforming Data	<ol style="list-style-type: none"> 1. Sterility and/or viability controls do not yield expected results. The mean log density for numbers control plates falls outside the specified range of 7.0-8.0 log₁₀/mL. 2. 3. Manage non-conforming data as specified in the study protocol; procedures are consistent with SOP ADM-07, Non-Conformance Reports. 4. <i>Retesting guidance:</i> For tests where the test chemical meets the performance standard and the numbers control mean log₁₀ density value is above 8.0, no retesting is necessary. For tests where the test chemical fails to meet the performance standard and the numbers control mean log₁₀ density is below 7.0, no retesting is necessary.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	<ol style="list-style-type: none"> 1. Strict adherence to the protocol is necessary for the validity of the test results. 2. Complete plating within 1 hour after the initiation of serial dilutions. 3. For spread plating: ensure that the entire surface of the agar plate is dry before adding inoculum. If necessary, leave the agar plates uncovered in the biological safety cabinet (BSC) until the moisture has been completely absorbed into the medium. 4. Use diluted test chemical within three hours of preparation unless

	specified otherwise.
11. Special Apparatus and Materials	<ol style="list-style-type: none"> 1. <i>Test organisms. Escherichia coli</i> (ATCC No. 11229) and <i>Staphylococcus aureus</i> (ATCC No. 6538) obtained from a reputable supplier (e.g., ATCC). 2. <i>Culture media.</i> Note: Commercial dehydrated media made to conform to the recipes provided in AOAC Method 960.09 may be substituted unless indicated otherwise. <ol style="list-style-type: none"> a. <i>Trypticase Soy Agar (TSA).</i> Prepare according to manufacturer’s instructions. Used for the generation of frozen stock cultures for <i>S. aureus</i>. b. <i>Tryptic Soy Broth (TSB).</i> Prepare according to manufacturer’s instructions. Used for the generation of frozen stock cultures for <i>S. aureus</i>. c. <i>Nutrient broth (NB):</i> Boil 5 g beef extract (powder), 5 g NaCl, and 10 g peptone (anaton) in 1 L H₂O for 20 minutes and dilute to volume with de-ionized water; adjust to pH 6.8±0.1. Filter through paper (Whatman No. 4, or equivalent). Steam-sterilize 20 min at 121°C. Used for the preparation of Nutrient agar (from AOAC method) plates and for the generation of <i>E. coli</i> frozen stock cultures. d. <i>Nutrient agar (NA; from AOAC method):</i> Dissolve Bacto agar to 1.5% (w/v) in nutrient broth (see section 11.2c) and adjust to pH 7.2-7.4. Steam-sterilize for 20 min at 121°C. Dispense into plates. Used for the generation of <i>E. coli</i> frozen stock cultures. e. <i>Nutrient agar – A (NA-A):</i> Boil 3 g beef extract, 5 g peptone, and 15 g salt-free agar in 1 L de-ionized water. <u>Do not use premixed dehydrated medium.</u> Dispense 10 mL portions in 20 × 150 mm tubes or 20 mL portions in 25 × 150 mm tubes and steam-sterilize for 20 min at 121°C. Slant tubes after sterilization and let cool. Used for daily transfers of test cultures for both test organisms. f. <i>Nutrient agar – B (NA-B):</i> Boil 3 g beef extract, 5 g peptone, and 30 g salt-free agar in 1 L de-ionized water. <u>Do not use premixed dehydrated medium.</u> Steam-sterilize for 20 min at 121°C. Temper medium prior to dispensing 20-30 mL portions into sterile Petri dishes. Used for development of the final test culture for both test organisms. 3. <i>Subculture media:</i> choose the appropriate recovery agar and neutralizer to inactivate the test chemical, for example: <ol style="list-style-type: none"> a. <i>Tryptone glucose extract agar (TGEA):</i> Prepare according to the

	<p>manufacturer's instructions. Used for the enumeration of numbers control samples for both test organisms.</p> <p>b. <i>Tryptone glucose extract agar plus Neutralizer (TGEA-N)</i>: Combine 24 g of dehydrated TGEA medium with 975 mL de-ionized water and 25 mL Neutralizer stock solution (see 11.4.b). Steam-sterilize for 15 min at 121°C. Used for the recovery of both test organisms from treated samples.</p> <p>4. <i>General Media and Reagents</i>:</p> <p>a. <i>TSB with 15% (v/v) glycerol</i>. Suspend 7.5 g tryptic soy broth in 212.5 mL de-ionized water. Add 37.5 mL glycerol and stir, warm slightly to dissolve. Dispense into bottles and autoclave for 15 min at 121°C. Store at room temperature for up to three months. Used for the preservation of frozen stock cultures as cryoprotectant solution.</p> <p>b. <i>Neutralizer stock solution (NSS)</i>: Mix 40 g Lecithin (granular), 280 mL polysorbate 80, and 1.25 mL 0.25 M phosphate buffer stock solution. Dilute with de-ionized water to 1 L and adjust pH to 7.2. Dispense in 100 mL portions and steam-sterilize for 20 min at 121°C. Used for the preparation of the Neutralizer blanks.</p> <p>c. <i>Neutralizer blanks (NB)</i>: Mix 100 mL neutralizer stock solution, 25 mL 0.25 M phosphate buffer stock solution, and 1675 mL of de-ionized water. Dispense into appropriate size vessel and steam-sterilize for 20 min at 121°C. Alternate neutralizers may be used as necessary.</p> <p>d. <i>0.25M Phosphate buffer stock solution (PBSS)</i>: Dissolve 34 g KH_2PO_4 in 500 mL de-ionized water in 1 L volumetric flask. Adjust pH to 7.2 with 1 N NaOH and dilute to 1 L volume mark. Filter sterilize. Used for the preparation of phosphate buffer solution.</p> <p>e. <i>Phosphate buffer dilution water (PBDW)</i>: Add 1.25 mL of PBSS to 1 L de-ionized water using a volumetric flask, mix thoroughly. Dispense into appropriate size vessel and steam sterilize for 20 min at 121°C. Used for numbers control assay and dilution blanks.</p> <p>f. <i>Tween-80 (polysorbate 80)</i>. Used for the preparation of PBS with 0.1% Tween 80.</p> <p>g. <i>PBS 10X solution</i></p> <p>h. <i>PBS (1X) with 0.1% Tween 80 (PBS + T80)</i>: Add 100 mL PBS 10X solution and 1 mL Tween 80 to a volumetric flask; fill with de-ionized water to the 1000 mL mark and mix thoroughly. Filter sterilize. Used during test culture harvesting.</p>
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	<ul style="list-style-type: none"> i. <i>Sterile water</i>. De-ionized (DI), distilled water or water with equivalent quality for making reagent solutions and culture media. j. <i>Blood Agar plate (BAP)</i>. Commercially purchased Trypticase soy agar (TSA) with 5% sheep's blood agar plates. Used for the presumptive identification of the test microbes. k. <i>Mannitol Salt Agar (MSA)</i>. Combine 111 g of dehydrated medium with 1 L de-ionized water and mix thoroughly. Steam-sterilize for 15 min at 121°C. Used for the presumptive identification of the test microbe <i>S. aureus</i>. l. <i>Xylose lysine deoxycholate agar (XLD)</i>. Commercially purchased plates. Used for the presumptive identification of the test microbe <i>E. coli</i>. <p>5. <i>Equipment and Glassware</i>. For sanitizer efficacy and numbers control, use 250 mL wide mouth Erlenmeyer flasks. For measuring sanitizers and dilution blanks use 100 mL graduated cylinders. For glassware used to prepare test chemical, refer to SOP MB-22.</p> <ul style="list-style-type: none"> a. <i>Sterile Petri dishes</i>. 20 mm × 100 mm in size. b. <i>Recirculating chiller unit and associated water bath</i>. For maintaining specified temperature of the test chemical (capable of maintaining 25 ± 1°C). c. <i>Test tube racks</i>. Any convenient style. d. <i>Transfer loops</i>. Make 4 mm ID single loop at end of 50–75 mm (2–3 in.) Pt or Pt alloy wire No. 23 B&S gage or 4 mm loop fused on 75 mm (3 in.) shaft (available from Johnson Matthey, West Chester, PA 19380, USA). Fit other end in suitable holder. Bend loop at 30° angle with stem. Commercially available 4 mm ID (i.e., 10 µL loops) transfer loops may also be used. Micropipettes may also be used instead of transfer loops. e. <i>Timer</i>. Certified timer for managing timed activities. f. <i>Micropipettes</i>. For performing culture transfers and serial dilutions. g. <i>Whatman No. 2 filter paper</i>. Sterile. h. <i>Gram stain kit</i>. i. <i>Vitek 2 Compact</i>. For microbe identification and confirmation.
<p>12. Procedure and Analysis</p>	<p>Prior to testing, perform the neutralization assay to determine if the prescribed neutralizer is appropriate for the test chemical (see section 12.9).</p>
<p>12.1 Culture</p>	<p>Refer to Attachment 2 for preparation of the frozen stock cultures. Reinitiate</p>

<p>Preparation</p>	<p>new frozen stock cultures every 18 months with a new lyophilized culture. Refer to MB-02: Tracking of Test Microorganisms, Section 12, for test microbe tracking and transfer notations.</p> <ol style="list-style-type: none"> a. For a daily culture, defrost a single cryovial of frozen stock culture at room temperature and briefly vortex to mix. Streak one loopful of the thawed frozen stock onto a NA-A slant and incubate at 36±1°C for 24±2 h. Only one daily transfer is required prior to the initiation of the final test culture. b. For the final test culture, add 5 mL of PBDW to a NA-A slant (daily culture). Using a sterile loop, dislodge growth from agar surface. Collect mixture and transfer to a flask containing 99 mL of PBDW. Mix thoroughly. Add 200 µL of the mixture to inoculate a minimum of 5 NA-B plates to create a bacterial lawn. Incubate at 36±1°C for 24±2 h. c. Record all culture transfers on the Organism Culture Tracking Form (see section 14).
<p>12.2 Test Culture Harvesting</p>	<ol style="list-style-type: none"> a. After incubation, add a minimum of 5 mL of PBS + T80 to each plate. Using a sterile rod, gently dislodge culture from agar surface, avoid disrupting agar. Combine culture from all plates and mix thoroughly. b. Filter culture through sterile Whatman No. 2 filter paper using a vacuum source; collect filtered test culture into a sterile vessel. Note: filter paper can be pre-cut and sterilized prior to use to fit on filter apparatus as necessary. c. Standardize the filtered test culture as necessary using PBDW to achieve a final test culture microbe titer between 1.0×10^9 CFU/mL and 1.0×10^{10} CFU/mL (9-10 log₁₀/mL).
<p>12.3 Test chemical Sample Preparation</p>	<ol style="list-style-type: none"> a. Prepare test chemical sample per SOP MB-22. <ol style="list-style-type: none"> i. Test ready-to-use test chemicals as received; no dilution is required. ii. If hard water is required as the diluent, prepare synthetic hard water as described in SOP MB-30. b. Equilibrate water bath and allow it to come to 25±1°C or the temperature specified (±1°C). Prepare the test chemical dilutions within 3 h of performing the assay. c. Dispense 99 mL aliquots of the diluted test chemical or ready-to-use test chemical into 250 mL sterile wide mouth Erlenmeyer flasks.

	<p>Prepare one flask per test microbe for each test chemical to be tested.</p> <ul style="list-style-type: none"> d. Place flasks in the equilibrated water bath for approximately 10 min to allow test chemical to come to specified temperature. e. Record the temperature of the water bath and recirculating chiller (i.e., chiller unit display) before and after testing on the Germicidal and Detergent Sanitizing Action of Disinfectants Method Test Information and Culture Preparation Sheet (see section 14). f. In addition, prepare a similar flask containing 99 mL of PBDW to use for numbers control for each organism (see section 12.5).
<p>12.4 Treated Sample Test Procedure</p>	<p>Flasks containing test culture × test chemical are referred to as the treated samples.</p> <ul style="list-style-type: none"> a. Add 1 mL of test culture to the test flask as follows: b. Swirl flask, stopping just before suspension is added, creating enough residual motion of liquid to prevent pooling of suspension at the point of contact with test sample. c. Add suspension midway between center and the inner edge of the flask with tip of pipette slightly immersed in test solution. Avoid touching the neck or side of flask during addition. Swirl flask to thoroughly mix contents. d. At 30 ± 3 seconds after addition of the test culture, transfer a 1 mL aliquot from the test flask (test culture × test chemical) to a tube containing 9 mL neutralizer blank and mix well. This corresponds to 10^{-1} dilution tube. Record timed events on the Germicidal and Detergent Sanitizing Action of Disinfectants Method Time Recording Sheet for Transfers (see section 14). e. <i>Treated samples plating.</i> From 10^{-1} tube (i.e., 9 mL neutralizer tube inoculated with 1 mL of exposed culture from the test flask), plate four 1 mL aliquots and four 0.1 mL aliquots onto TGEA-N plates, for a total of 8 plates per treated sample. This will result in 10^{-1} and 10^{-2} dilutions, respectively. Incubate plates at $36 \pm 1^\circ\text{C}$ for 24-30 hours. f. Following incubation, count colonies on TGEA-N plates. Counts over 300 are recorded as TNTC. Record plate counts on the Germicidal and Detergent Sanitizing Action of Disinfectants Method Results Sheet (see section 14).
<p>12.5 Numbers Control</p>	<p>Flask containing 99 mL of PBDW is used for the numbers control procedure and referred to as the numbers control sample.</p>

<p>Procedure</p>	<ol style="list-style-type: none"> a. Conduct numbers control procedure within 5 minutes of completion of treated samples. b. In a sterile 250 mL wide mouth Erlenmeyer flask containing 99 mL sterile PBDW, add 1 mL of the test culture (same test culture used for the treated samples) as follows: c. Swirl flask, stopping just before suspension is added, creating enough residual motion of liquid to prevent pooling of suspension at the point of contact with test sample. d. Add suspension midway between center and the inner edge of the flask with tip of pipette slightly immersed in test solution. Avoid touching to the neck or side of flask during addition. Swirl flask to thoroughly mix contents. e. <i>Numbers control plating.</i> Within 30 seconds of addition of test culture, transfer a 1 mL aliquot from the numbers control flask (test culture × PBDW) into a tube containing 9 mL of neutralizer and mix well. This corresponds to 10⁻¹ dilution tube. Make serial 10-fold dilutions in 9 mL PBDW, out to 10⁻⁶. f. Plate four 1 mL aliquots and four 0.1 mL aliquots from the 10⁻⁶ dilution tube onto TGEA plates, for a total of 8 plates per numbers control sample. This will result in 10⁻⁶ and 10⁻⁷ dilutions, respectively. g. Incubate plates at 36±1°C for 24-30 hours. h. Following incubation, count colonies on TGEA plates. Counts over 300 are recorded as TNTC. Record plate counts on the Germicidal and Detergent Sanitizing Action of Disinfectants Method Results Sheet (see section 14).
<p>12.6 Sterility controls</p>	<ol style="list-style-type: none"> a. Neutralizer blank – plate 1 mL from a previously unopened neutralizer tube onto a TGEA plate. b. Test chemical – plate 1 mL of the test chemical used in the assay onto a TGEA plate. c. Diluent – plate 1 mL of the diluent used for the preparation of the test chemical, if necessary, onto a TGEA plate. d. Incubate all plates at 36±1°C for 24-30 hours, record results. e. To be considered valid, no growth should be observed on any of the sterility controls.
<p>12.7 Results</p>	<ol style="list-style-type: none"> a. For a valid test, the mean numbers control counts must fall between 7.0-8.0 log₁₀/mL.

	<p>b. For the test chemical to be considered efficacious, a mean LR ≥ 5 is required with a contact time of 30 seconds.</p>
<p>12.8 Confirmatory Steps for Test Microbes</p>	<p>a. Conduct presumptive identification of the test microbes when results are indicative of a failing efficacy evaluation or when results are inconclusive.</p> <p>b. Confirm representative growth from one plate per treatment sample by Gram stain and growth characteristics on general and selective media.</p> <p>c. Gram stains are performed on smears taken from the treatment sample plates. For the additional confirmatory tests, a smear of culture from each selected treatment sample plate is streaked onto BAP and selective media appropriate for the test organism and incubated for 24 ± 2 hr at $36 \pm 1^\circ\text{C}$. See Attachment 1 for Gram stain reactions, cell morphology, and colony characteristics on solid media.</p> <p>d. If characteristics on general and/or selective media are unusual, then further confirmation will be conducted by VITEK, see SOP-QC-22: VITEK 2 Compact: Use, Maintenance and Quality Control Procedures for details.</p> <p>e. If confirmatory testing determines that the identity of the organism was not the test organism, the entry on the results sheet must be annotated to indicate a contaminant was present.</p>
<p>12.9 Neutralization Confirmation Test</p>	<p>a. Perform a neutralization confirmation test prior to or concurrently with the sanitizer evaluation assay. The neutralization assay must demonstrate the recovery of a low-level test organism population (e.g., 10-100 CFU/mL) on the recovery media (i.e., TGEA-N and TGEA).</p> <p>b. <i>Test Culture Titer (TCT)</i>. Add 0.1 mL of the test organism, which has been serially diluted to target between 10-100 CFU/mL to 10 mL of PBDW and mix thoroughly. Dilutions 10^{-4} and 10^{-5} should provide the range of 10-100 CFU/mL. Hold the mixture for a minimum of two minutes. Plate 0.1 mL aliquots in duplicate onto TGEA. Incubate plates at $36 \pm 1^\circ\text{C}$ for 24-30 hours and record number of colonies.</p> <p>c. <i>Neutralization Confirmation Treatment (NCT)</i>. Add 1 mL of the test chemical to 9 mL of the prescribed neutralizer and mix thoroughly. Within 30 seconds, inoculate the sample with 0.1 mL of the test organism used for the TCT. Mix thoroughly. Hold the mixture for a minimum of two minutes. Plate 0.1 mL aliquots in duplicate onto</p>

	<p>TGEA-N. Incubate plates at 36±1°C for 24-30 hours and record number of colonies.</p> <p>d. <i>Neutralization Toxicity Treatment (NTT)</i>. Add 0.1 mL of the test organism used for the TCT to 10 mL of the prescribed neutralizer and mix thoroughly. Hold the mixture for a minimum of two minutes. Plate 0.1 mL aliquots in duplicate onto TGEA-N. Incubate plates at 36±1°C for 24-30 hours and record number of colonies.</p> <p>e. Plates that have colony counts over 300 are reported as TNTC. Record the counts on the Neutralization Confirmation Assay Results Sheet (see section 14).</p> <p>f. <i>Neutralization Results and Calculations</i>. In order to demonstrate effective neutralization of the sanitizer, differences between treatments should not exceed 1.0 log (e.g., TCT minus NCT).</p> <p>g. <i>To calculate CFU/mL for Neutralization Confirmation Test use the following equation.</i></p> $CFU/mL = \frac{\{(Mean\ CFU\ for\ 10^{-x}) + (Mean\ CFU\ for\ 10^{-y})\}}{10^{-x} + 10^{-y}}$ <p>where 10^{-x} and 10^{-y} are the dilutions plated. Two plates per dilution are plated for all treatments. Alternatively, if only one dilution is plated, use the same equation for only one dilution. Use counts of 0 to 300 for calculation purposes. Score counts >300 as TNTC (too numerous to count).</p> <p>NOTE: A spreadsheet will be used for data analysis and calculations associated with the neutralization confirmation assay.</p>
<p>13. Data Analysis/ Calculations</p>	<p>1. Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Both electronic and hard copies of the spreadsheet will be retained.</p> <p>2. To calculate CFU/mL, use the following equation.</p> $CFU/mL = \frac{\{(CFU\ for\ 10^{-x}) + (CFU\ for\ 10^{-y})\}}{10^{-x} + 10^{-y}}$ <p>where 10^{-x} and 10^{-y} are the dilutions plated. Four plates per dilution are plated for treated samples and numbers control samples. Use counts of 0 to 300 for calculation purposes. Score counts >300 as TNTC (too numerous to count).</p> <p>3. Calculate the mean log₁₀ density (LD) for numbers control plates.</p> <p>4. Calculate the mean log₁₀ density (LD) for treated samples plates.</p> <p>5. Calculate the log₁₀ reduction (LR) for treated samples:</p> <p>Log₁₀ reduction = mean log₁₀ numbers control – mean log₁₀ treated sample</p>

	<p>NOTE: If no growth is observed for treated sample, substitute 0.5 at the lowest (least dilute) dilution and account for the dilution factor in the calculations.</p>																						
<p>14. Forms and Data Sheets</p>	<ol style="list-style-type: none"> 1. Attachment 1: Typical Growth Characteristics of strains of <i>S. aureus</i> and <i>E. coli</i> 2. Attachment 2: Culture Initiation Flow Chart for <i>S. aureus</i> and <i>E. coli</i> 3. Test Sheets. Test sheets are stored separately from the SOP under the following file names: <table border="0" style="width: 100%; margin-left: 40px;"> <tr> <td style="width: 70%;">Organism Culture Tracking Form</td> <td style="text-align: right;">MB-27-04_F1.docx</td> </tr> <tr> <td>Test Microbe Confirmation Sheet (Quality Control)</td> <td style="text-align: right;">MB-27-04_F2.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of Disinfectants Method: Test Information and Culture Preparation Sheet</td> <td style="text-align: right;">MB-27-04_F3.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of Disinfectants Method: Serial Dilution/Plating Tracking Form</td> <td style="text-align: right;">MB-27-04_F4.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of Disinfectants Method: Titer of Final Test culture Form</td> <td style="text-align: right;">MB-27-04_F5.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of Disinfectants Method: Results Sheet</td> <td style="text-align: right;">MB-27-04_F6.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of Disinfectants Method: Time Recording Sheet for Transfers</td> <td style="text-align: right;">MB-27-04_F7.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of Disinfectants Method: Test Microbe Confirmation Sheet</td> <td style="text-align: right;">MB-27-04_F8.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Confirmation Assay – Neutralization Confirmation Control</td> <td style="text-align: right;">MB-27-04_F9.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Toxicity Control</td> <td style="text-align: right;">MB-27-04_F10.docx</td> </tr> <tr> <td>Germicidal and Detergent Sanitizing Action of</td> <td style="text-align: right;">MB-27-04_F11.docx</td> </tr> </table> 	Organism Culture Tracking Form	MB-27-04_F1.docx	Test Microbe Confirmation Sheet (Quality Control)	MB-27-04_F2.docx	Germicidal and Detergent Sanitizing Action of Disinfectants Method: Test Information and Culture Preparation Sheet	MB-27-04_F3.docx	Germicidal and Detergent Sanitizing Action of Disinfectants Method: Serial Dilution/Plating Tracking Form	MB-27-04_F4.docx	Germicidal and Detergent Sanitizing Action of Disinfectants Method: Titer of Final Test culture Form	MB-27-04_F5.docx	Germicidal and Detergent Sanitizing Action of Disinfectants Method: Results Sheet	MB-27-04_F6.docx	Germicidal and Detergent Sanitizing Action of Disinfectants Method: Time Recording Sheet for Transfers	MB-27-04_F7.docx	Germicidal and Detergent Sanitizing Action of Disinfectants Method: Test Microbe Confirmation Sheet	MB-27-04_F8.docx	Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Confirmation Assay – Neutralization Confirmation Control	MB-27-04_F9.docx	Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Toxicity Control	MB-27-04_F10.docx	Germicidal and Detergent Sanitizing Action of	MB-27-04_F11.docx
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Germicidal and Detergent Sanitizing Action of Disinfectants Method: Titer of Final Test culture Form	MB-27-04_F5.docx																						
Germicidal and Detergent Sanitizing Action of Disinfectants Method: Results Sheet	MB-27-04_F6.docx																						
Germicidal and Detergent Sanitizing Action of Disinfectants Method: Time Recording Sheet for Transfers	MB-27-04_F7.docx																						
Germicidal and Detergent Sanitizing Action of Disinfectants Method: Test Microbe Confirmation Sheet	MB-27-04_F8.docx																						
Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Confirmation Assay – Neutralization Confirmation Control	MB-27-04_F9.docx																						
Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Toxicity Control	MB-27-04_F10.docx																						
Germicidal and Detergent Sanitizing Action of	MB-27-04_F11.docx																						

	<p>Disinfectants Method: Neutralization Confirmation Assay – Test culture Control</p> <p>Germicidal and Detergent Sanitizing Action of MB-27-04_F12.docx Disinfectants Method: Neutralization Confirmation Assay Results Sheet</p>
15. References	<ol style="list-style-type: none">1. Official Methods of Analysis. Revised 2013. AOAC INTERNATIONAL, Gaithersburg, MD (Method 960.09).2. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. <i>Bergey's Manual of Systematic Bacteriology Volume 2</i>. Williams & Wilkins, Baltimore, MD. <i>E. coli</i> p. 420; <i>S. aureus</i> p. 1015.

Attachment 1

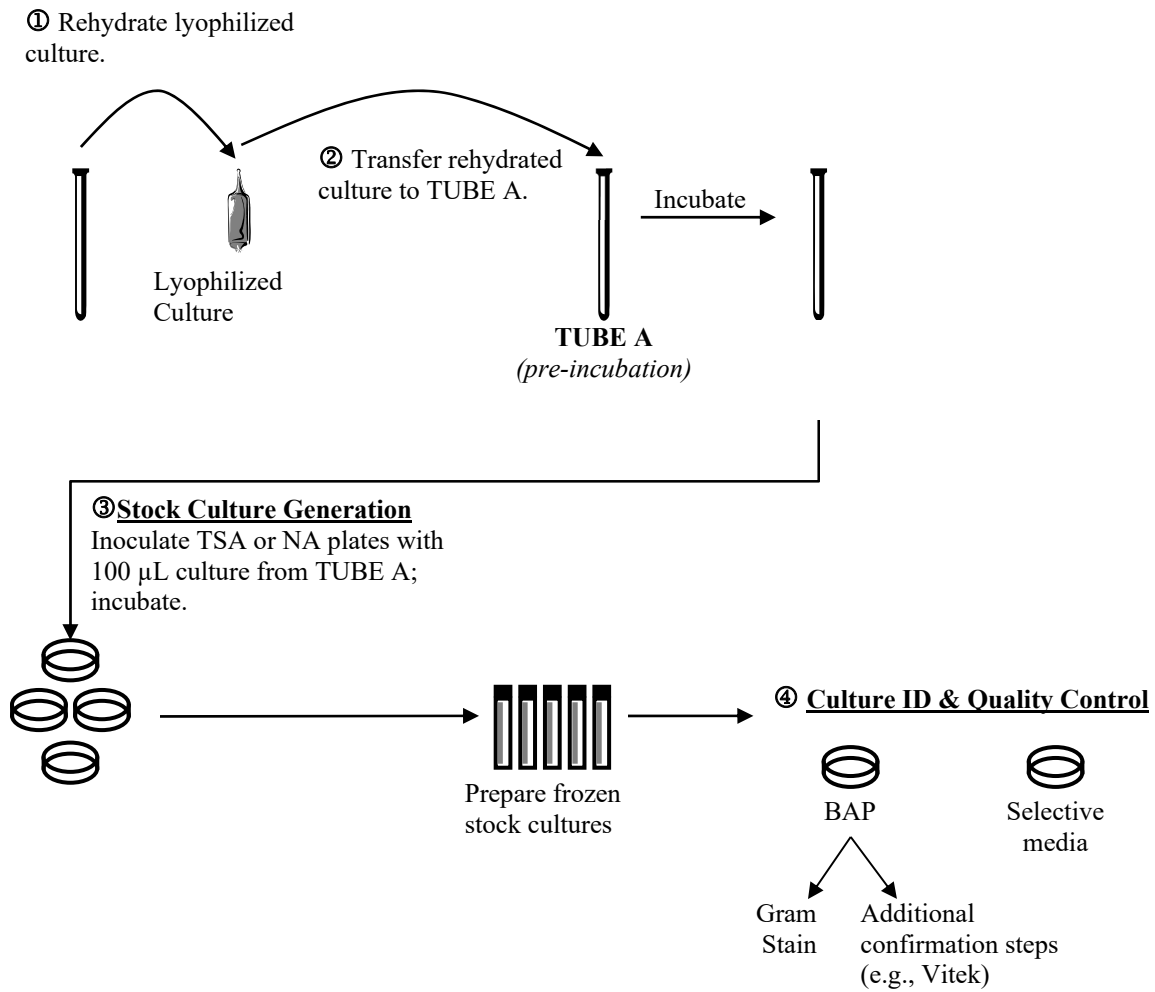
Typical Growth Characteristics of strains of *S. aureus* and *E. coli* (see reference 15.2)

	<i>S. aureus</i> *	<i>E. coli</i> *
Gram stain reaction	Gram positive (+)	Gram negative (-)
Typical Growth Characteristics on Solid Media		
BAP	small, circular, yellow or white, glistening, beta hemolytic	large, round, white colonies, non-hemolytic.
Mannitol Salt agar	circular, small, yellow colonies, agar turning fluorescent yellow	No Growth
XLD agar	No growth	large, flat, yellow colonies with no black center.
Typical Microscopic Characteristics		
Cell dimensions	0.5-1.5 µm in diameter	0.5 × 1-3 µm in diameter
Cell appearance	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	varying from almost coccoid forms to long rods, occurring singly, in pairs and in short chains. Motile or non-motile, if motile with flagella.

*After 24±2 hours incubation

Attachment 2

Culture Initiation and Stock Culture Generation Flow Chart for *S. aureus* and *E. coli*.



Preparation of Frozen Stock Cultures. Refer to SOP MB-02 for establishment of the organism control number.

- a. Initiate new stock cultures from lyophilized cultures of *Staphylococcus aureus* (ATCC No. 6538) and *Escherichia coli* (ATCC No. 11229) from ATCC or a reputable supplier within 18 months.
- b. Rehydrate lyophilized culture.
 - i. Open ampule of freeze-dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of TSB for *S. aureus* and 5-6 mL of NB for *E. coli*, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth designated as "TUBE A." Mix well.
 - ii. For lyophilized cultures received on loops/swabs, as pellets etc., open and rehydrate the lyophilized organism as indicated by the manufacturer. Transfer the rehydrated culture to a tube of 5-6 mL of TSB for *S. aureus* and 5-6 mL of NB for *E. coli* (e.g., swish inoculated swab/loop in the TSB/NB tube or transfer the rehydrated pellet to the TSB/NB tube); TSB/NB tube is designated as "TUBE A".
 - iii. Incubate broth culture (TUBE A) at $36\pm 1^{\circ}\text{C}$ for 24 ± 2 h. Record all manipulations on the Organism Culture Tracking Form (see section 14).
- c. Following incubation, use a sterile spreader to inoculate a sufficient number of TSA plates for *S. aureus* or NA plates for *E. coli* (e.g., 5 to 10 plates per organism) with 100 μL each of the 24 ± 2 h culture. Incubate plates at $36\pm 1^{\circ}\text{C}$ for 24 ± 2 h.
 - i. For QC purposes, perform a streak isolation of the 24 ± 2 h broth culture on a BAP. In addition, for *S. aureus*, streak a loopful onto selective media (MSA); for *E. coli*, streak a loopful onto XLD. Incubate all plates at $36\pm 1^{\circ}\text{C}$ for 24 ± 2 h.
- d. Following incubation, add 5 mL cryoprotectant solution (TSB with 15% v/v glycerol) to the surface of each agar plate. Re-suspend the cells in this solution using a sterile spreader or a sterile swab and aspirate the cell suspension from the surface of the agar. Transfer the suspension into a sterile vessel. Repeat by adding another 5 mL of cryoprotectant to the agar plates, re-suspend the cells, aspirate the suspension and pool with the initial cell suspension.
 - i. For QC purposes, use the pooled suspension to perform a streak isolation on a BAP. In addition, for *S. aureus*, streak a loopful onto selective media (MSA); for *E. coli*, streak a loopful onto XLD. Incubate all plates at $36\pm 1^{\circ}\text{C}$ for 24 ± 2 h. Continue QC steps as per sections g through i.
- e. Mix the pooled contents of the vessel thoroughly. Immediately after mixing, dispense approximately 0.5 to 1.0 mL aliquots into cryovials (e.g., 1.5 mL cryovials).
- f. Place and store the cryovials at -70°C or below; these are the frozen stock cultures. Stock cultures may be used up to 18 months; reinitiate using a new lyophilized

culture. These cultures are single use only.

- g. Following the incubation period (see d.i), record the colony morphology as observed on the BAPs and selective media plates (including the absence of growth). See Attachment 1 for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.
- h. For each organism, perform a Gram stain and Vitek from growth taken from the BAPs according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).
- i. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).